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Influence of Oxygen and pH on Methanethiol Production from L-Methionine by *Brevibacterium linens* CNRZ 918

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The effects of dissolved oxygen concentration and pH on the growth of *Brevibacterium linens* CNRZ 918 and its production of methanethiol from L-methionine were investigated. Optimal specific methanethiol production was obtained at 25% saturation of dissolved oxygen and at a pH between 8 and 9, whereas optimal cell growth occurred at 50% oxygen saturation and when the pH was maintained constantly at 7. Methanethiol production by nonproliferating bacteria required the presence of L-methionine (7 mM) in the culture medium. This was probably due to the induction of enzyme systems involved in the process. The intracellular concentration of L-methionine seemed to play a key role in this process. *B. linens* CNRZ 918 tolerated alkaline pHs with a maximal growth pH of approximately 9. Its orange pigmentation seemed to depend on the presence of L-methionine in the culture medium and on the concentration of dissolved oxygen.

Aromatic sulfur compounds are responsible for the typical "garlicky" character of some processed or soft cheeses with washed or blooming rinds, for which surface flora intervene. Methanethiol appears to be the precursor molecule of most aromatic sulfur compounds in cheeses, via chemical (4, 5) or microbial (9, 10, 20) condensation reactions. Methanethiol has been shown to arise from microbial degradation, which is also the case for microorganisms isolated from cheeses (11, 13, 19, 28, 29). Among the aerobic microorganisms playing an important role in the ripening of washed-rind orange cheeses are the orange coryneform bacteria present on the surface which are related to *Brevibacterium linens* (red ferment of cheeses). The bacteria of this species represent the predominant flora and probably participate in the formation of the intense garlicky odor (putrid cheese type). They are also present in large numbers in other cheeses, e.g., Comté (1) and Camembert (26). Most of the orange strains isolated from these two types of cheeses, either belonging or related to *B. linens*, can demethiolate methionine (13a).

A large body of published data exists concerning the effect of oxygen on lysine biosynthesis (17, 25, 27, 31) and glutamic acid biosynthesis (7, 12, 14, 15, 22, 24, 33, 34) by species relatively close to *B. linens*. On the other hand, the effect of oxygen on the catabolism of L-methionine, especially its demethiolation, has apparently never been examined either in *B. linens* or in most other producers of methanethiol isolated from cheeses or from other sources.

We recently examined methanethiol production from L-methionine by a strain of *B. linens* grown in noncontrolled conditions in a complex medium (11). In this report, we present additional data on methanethiol production from L-methionine under controlled conditions of dissolved oxygen concentration and pH with the same strain grown in a fermentor in a more chemically defined medium.

MATERIALS AND METHODS

Organism. *B. linens* CNRZ 918 was obtained from the culture collection of the Centre National de Recherches Zootechniques. The strain was originally isolated by Accolas et al. (1) from the surface rind of Comté cheese. It is an obligate aerobe and forms orange colonies.

Culture medium. The basic medium (BM) had the following composition, in grams liter^{-1}: sodium DL-lactate (Sigma Chemical Co., St. Louis, Mo.), 5.0; yeast extract (Difco Laboratories, Detroit, Mich.), 1.0; peptone (Bio-case low salt; Mérieux, Marcy l'Etoile, France), 1.0; L-alanine (Sigma), 5.0; K_2HPO_4, 1.7; KH_2PO_4, 0.24; $CaCl_2$, 0.025; $MgSO_4 \cdot 7H_2O$, 0.2; NaCl, 10. The BM was supplemented with L-methionine (Sigma) 1.0, to form BMM.

When cultures with noncontrolled pH were used, the pH was adjusted with 10 N NaOH (Prolabo, Paris, France) to 7.8 before autoclaving. In the case of controlled pH cultures, the corresponding values were 7.2, 8.2, or 9.2 (the pH of the media decreased by about 0.2 U after sterilization).

Cell fermentor. A 15-liter fermentor (Biolafitte, Saint Germain en Laye, France) was used in this work. It was equipped with systems for controlling temperature, mixing speed, dissolved oxygen (Biolafitte O_2 probe) and included a refrigerated sampler (Braun). A fuller description of the system and parameter control was published by Tayeb et al. (32).

Cultures. In order to ensure repeatability, a stock of frozen cultures was constituted by inoculating a series of 20-ml tubes of complex medium (11) with 5% *B. linens* with no incubation, followed by freezing at −20°C, conditions compatible with growth ability and methanethiol production capacity (11). At the start of each experiment, one 20-ml culture tube was thawed, transferred to a 100-ml Erlenmeyer flask, and incubated at 26°C in a reciprocal shaker (100 cycles per min) for 24 h. A 1-ml amount of this culture was used to inoculate the preculture (100 ml of BMM in a 500-ml Erlenmeyer flask) which had been grown with shaking for 70 h. The latter culture was used to inoculate the fermentor.

Sterilizing the medium, inoculation, supply of dissolved oxygen, and control of pH. The fermentor and its accessories (input and output filters, condenser, and valves) were steam sterilized for 40 min at 120°C. The fermentation vessel was then filled with 10 liters of BM or BMM containing antifoam (Rhodorsil 426 R silicone, Prolabo; final concentration, 1%), sterilized for 20 min at 120°C, and then cooled to 26°C. The 10 liters of BM or BMM in the vessel were then inoculated

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with 100 ml of culture. All cultures were grown at 26°C with stirring (250 rpm). Dissolved oxygen was supplied by circulating a mixture of N_2 (R grade, l'Air Liquide) and compressed air. The nitrogen flow rate was held constant, and the flow rate of the compressed air was varied with a motorized valve to maintain the dissolved oxygen concentration constantly at the chosen value, with reference to a 100% level obtained by saturating the sterilized medium before inoculation.

In the case of cultures at constant pH and constant dissolved O_2 at 50% saturation, pH was controlled by adding 10 N NaOH and 2.5 N HCl or 15 N phosphoric acid (all from Prolabo). Acid and base were aseptically added dropwise from the top of the vessel by peristaltic pumps controlled by the pH control module.

Growth and morphology. Amino acid analysis in the culture medium proceeded by the following steps. Samples (20 ml) of medium were removed every 2 h with an automatic sampler. After centrifugation (12,500 × *g*, 20 min) the supernatant was aseptically filtered (200-nm pore size) and then diluted in citrate buffer, pH 2.2. This solution was analyzed with a Biotronik LC 5000 automatic amino acid analyzer. Cell morphology was noted by observation with a phase-contrast microscope, and A_{650} was determined by using a Beckman 25 spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). The pH of the cultures was recorded continuously.

Methanethiol production capacity determination by culture. Methanethiol production capacity (MTPC) was determined as follows. Methanethiol produced by the culture was removed by the flow of the gas mixture and assayed at the fermentor output filter. It was trapped by bubbling the gas for 2 min in 3 ml of 5% mercuric acetate (Aldrich Chemical Co., Inc., Milwaukee, Wis.) in 10% acetic acid (E. Merck AG, Darmstadt, Federal Republic of Germany). Methanethiol was then assayed by the method of Sliwinsky and Doty (30). The mercaptide salt concentration was determined by referring to a standard curve generated with ethanethiol.

MTPC determination with nonproliferating cells. Cells from 20 ml of culture were harvested by centrifuging (Ivan Sorvall, Inc., Norwalk, Conn.) at 12,500 × *g* for 20 min at 4°C. The pellet was washed with 20 ml of 50 mM Tris hydrochloride (Trizma, Sigma; pH 8.0) at 4°C and suspended in the same buffer. The remainder of the assay was carried out as described elsewhere (11), except that the final concentration of L-methionine used was 50 mM.

The specific MTPC (sMTPC) of the cells was defined as nanomoles of methanethiol produced second^{-1} gram of dry weight of cells^{-1} (nanokatals gram of dry weight^{-1}) from L-methionine under the specific conditions of the assay.

Transport of L-methionine. The technique used was the same as previously described (6) for aromatic amino acids, except that L-[3,4-^{14}C]methionine (Commissariat à l'Energie Atomique, France) was used.

All assays were done at least twice. When the assay with dissolved O_2 at 25% and nonregulated pH was used to produce cells, a variation of 5% was observed (for the mean of five cultures). The mean variations observed were always <10%.

RESULTS

Effect of concentration of dissolved oxygen. The sMTPC of nonproliferating cells varied as a function of the percent saturation of dissolved oxygen (Fig. 1b). It consistently attained a maximum level, obtained with different growth and culture pH values, and was correlated with the appearance of coccoid forms (the proportion of coccoid forms became >40% at the maximal sMTPC). Maximal sMTPC (96 nkat g^{-1}) was obtained with cells grown in 25% dissolved oxygen, when A_{650} was 2.8 and pH was 8.3. After 70 h of growth, the sMTPC decreased by about 50%, except in the case of 75% dissolved oxygen, for which a 75% decrease was observed.

The concentration of dissolved oxygen also affected the pigmentation of the culture as judged by visual observation. An intense orange color was obtained after 20 h at 50 or 75% saturation, whereas at 25% saturation, the same color was obtained after 38 h. At 12.5% saturation, the intense orange color did not appear. The MTPC of the culture, equivalent to thiols released by the fermentor, also varied as a function of dissolved oxygen concentration and of culture time (Fig. 1c). At 12.5 or 25% dissolved oxygen, the MTPC of the culture reached a maximum level within 30 h and subsequently remained constant. At 50 and 75%, however, production

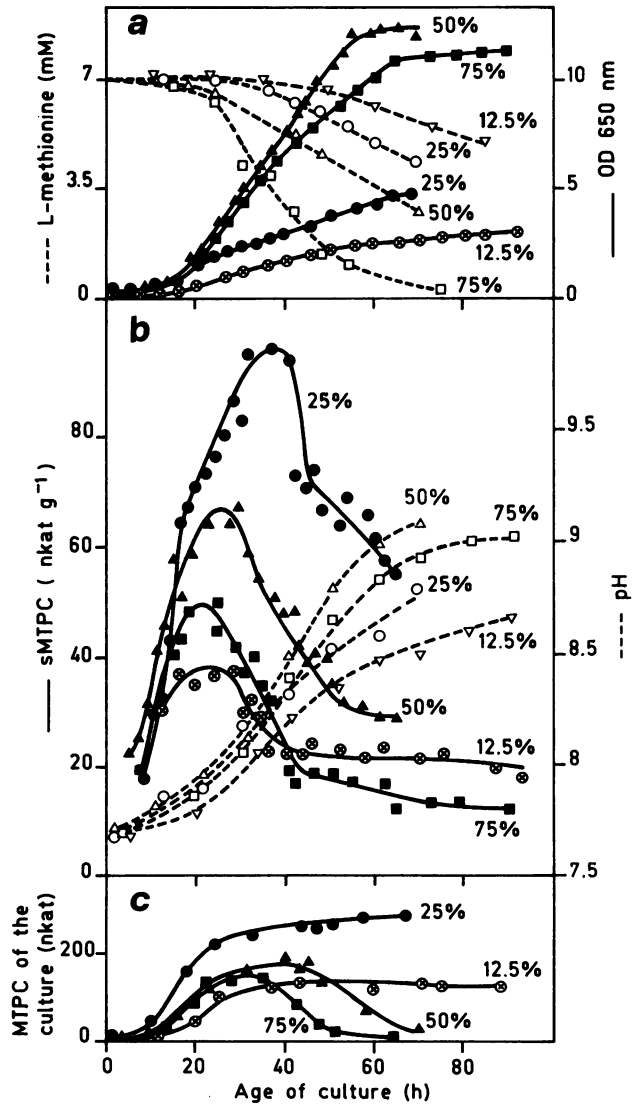

FIG. 1. Effect of dissolved oxygen concentration in BM plus methionine on growth and L-methionine concentration (a), on sMTPC of nonproliferating cells and culture pH (b), and on MTPC (c) in the *B. linens* culture.

attained a maximum and then decreased, more rapidly at 75%. The MTPC of the various cultures began its decline when the L-methionine concentration decreased to about 3 mM (Fig. 1a and c).

The utilization of L-methionine during growth was low until about 25 h and then increased in proportion to the concentration of dissolved oxygen (Fig. 1a). Thus, after 70 h in the presence of 12.5, 25, 50, and 75% saturation of dissolved oxygen, there was 80, 62, 40, and 7% residual L-methionine, respectively.

We also examined the utilization of other amino acids during growth. Arginine, glutamic acid, lysine, and alanine (in order of decreasing utilization) were all monitored. These rates of utilization, as well as growth and cell yield, were proportional to the concentration of dissolved oxygen in BMM (Fig. 1a). However, 75% O_2 saturation was a less favorable condition than was 50%, at least after 40 h of culture. This effect of O_2 concentration was not pronounced before 20 h of growth (except in the case of 12.5% saturation).

Growth was accompanied by an increased pH in the media, and the rate of this increase was greater in the 50% saturation culture than in the others. Thus, after 70 h, the pH of cultures were 8.50, 8.80, 9.1, and 8.75 at 12.5, 25, 50, and 75% O_2, respectively. The generation times obtained were 6 h at 12.5% and 5 h for other oxygen concentrations.

Ammonia production by the cultures varied, but was parallel with the increase in pH. Cell morphology differed, depending on the physiological phase of growth. Rod-shaped cells predominated during exponential growth, and cocci were the most frequently occurring type stationary phase.

Effect of culture pH. At noncontrolled pH and at pH 8 or 9, the maximal sMTPC of nonproliferating cells was identical (70 nkat g^{-1}) and occurred after the same time interval of 25 h (Fig. 2b). The sMTPC subsequently uniformly decreased until 60 h (in cultures with no pH control), whereas it remained at 70% of the maximum level after 40 h in cultures whose pH was controlled at 8 or 9. At controlled pH 7, the sMTPC occurred sooner and was lower than at pH 8 or 9, decreasing to negligible values after 40 h of growth. Although the proportion of cocci was variable in all the cultures, there was a considerable increase once the maximal sMTPC was reached. The appearance of the orange color was observed at maximal sMTPC.

Growth when dissolved oxygen was set at 50% and the pH was controlled at 7 or 8 was very close to that obtained without pH control (Fig. 2a). Cell yield was better at pH 7 than at pH 8, as a result of a shorter lag time. Growth at controlled pH 9, however, was much lower than at other pH values, and the final cell yield was 2.5 times lower.

Cocci predominated at pH values higher than 8.5 in noncontrolled cultures, but this was not noted in cultures controlled at pH 9.

Effect of adding L-methionine during growth. Before adding L-methionine (at 24.5 h), growth in BM and BMM was identical (Fig. 3a). The sMTPC of nonproliferating cells was low before the addition of L-methionine and subsequently underwent a considerable increase (Fig. 3b). The maximum was noted 25 h after adding L-methionine, although it remained 25% lower than that recorded in the BMM culture, which had its methionine added at time zero. Beyond this maximum, the sMTPC stabilized at 75% of the maximum level for at least 30 h, in contrast to stabilization at 50% saturation when L-methionine was added at time zero (Fig. 3b).

Pigmentation appeared about 2 h after the addition of

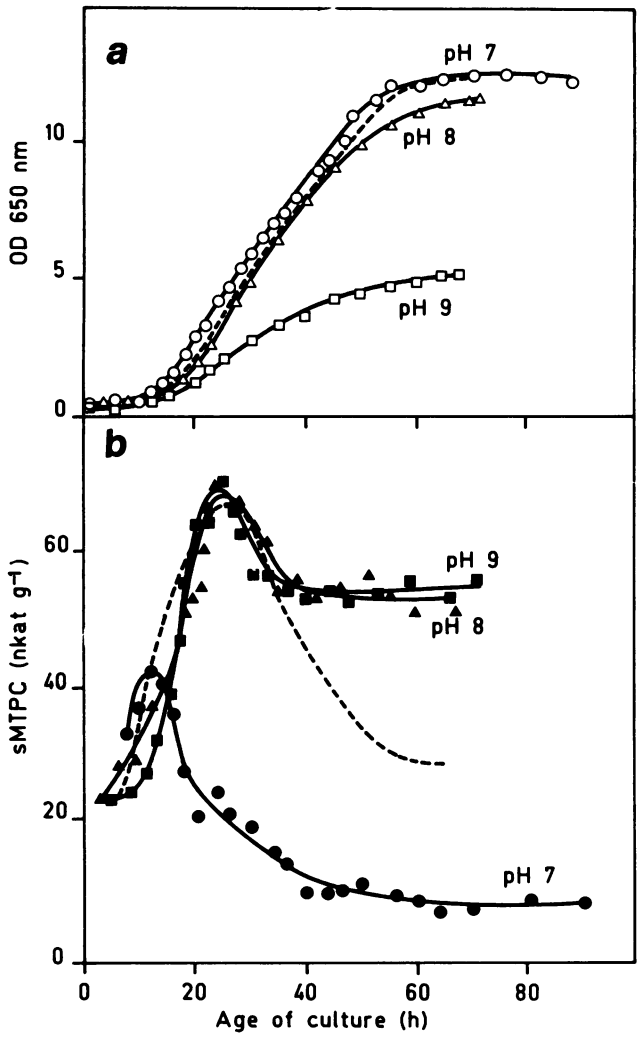

FIG. 2. Effect of culture pH in the presence of 50% dissolved oxygen on growth in BM plus methionine (a) and on the sMTPC of nonproliferating *B. linens* CNRZ 918 cells (b). -----, Growth and sMTPC obtained at noncontrolled pHs (curves are taken from Fig. 1).

L-methionine to BM. Maximal pigmentation occurred 25 h after this addition, an interval identical to that observed when L-methionine was initially present in the medium.

Culture samples of 100 ml were removed from the fermentor 30 min after the addition of L-methionine and were incubated in 500-ml Erlenmeyer flasks. Growth was similar to that observed in the fermentor, and final cell yield was only about 15% lower (Fig. 3a). The change in the sMTPC during this time, however, was quite different, with a lower maximum obtained more rapidly (13 h after adding L-methionine) which was marked by a uniform decrease with no plateau (Fig. 3b). This pattern was identical to that noted in BMM cultures (L-methionine at time zero) raised in agitated flasks. When a flask culture obtained from the fermentor was supplemented with 3.1 mM L-ethionine, final cell yield increased by 32%, with an identical growth profile until 40 h after culture and an sMTPC with a maximum 27% higher at the same age of culture as that of the control flask culture not receiving L-ethionine. Beyond this maximum, the sMTPC decreased more rapidly than it did in the control.

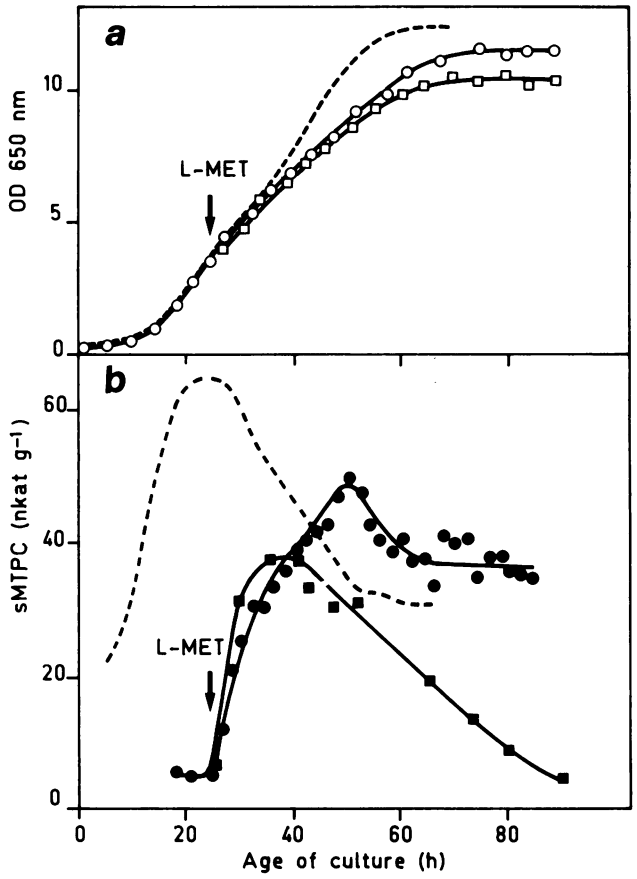

FIG. 3. Effect of the addition of L-methionine on growth (a) and sMTPC of nonproliferating *B. linens* CNRZ 918 cells (b). (○, ●), Cells grown in the fermentor at 50% saturation of dissolved oxygen; (□, ■), cells transferred to flasks after the addition of L-methionine. Arrow, time of L-methionine (7 mM final concentration) addition to the BM in the fermenter. ---, Growth and sMTPC obtained when L-methionine was present in the medium at the moment of inoculation (curves are taken from Fig. 1).

When 4.1 mM L-cysteine was added to the medium, cell yield decreased by 10%, and the sMTPC increased (slightly) by 8%. The addition of other amino acids (6.6 mM Gly, 5.6 mM Ala, 4.7 mM Ser, 3.8 mM Asn, 3.4 mM Gln, 5.6 mM Ala + 6.6 mM Gly, and 4.7 mM Ser + 6.6 mM Gly) had no effect on growth or on the sMTPC.

DISCUSSION

As is often observed for the production of other metabolites, methanethiol production was maximal under growth conditions which were not the most favorable, i.e., maximal production at 25% O_2 saturation with pH controlled at 8 or 9 or maximal growth at 50% O_2 saturation with pH either controlled at 7 or not controlled.

The specific production of methanethiol required the presence of L-methionine in the culture medium. The time required for its maximum induction (~25 h after L-methionine addition) corresponds to an enzyme induction. Since the transport has been shown to be constitutive (uptake of L-methionine was 9 nkat g^{-1} at pH 8), only the L-methionine-γ-demethiolase was inducted, thus confirming our earlier data (11). The sMTPC in *B. linens* CNRZ 918 decreased after significant proportions of coccoid forms appeared in the culture. Our results indicate that this decreased sMTPC was primarily caused by the decreasing levels of L-methionine in the medium, which influenced the intracellular concentration. 75% oxygen than at 50%, the sMTPC of the nonproliferating cells was lower, and growth was identical. This signifies that methionine entry is not limited. With regard to the sMTPC determination test, carried out under standard conditions with nonproliferating cells (see the Materials and Methods section), the effect of oxygen on the sMTPC can be only a long-lasting inactivation effect; oxygen would participate either in the regulation of the induction of enzymes involved in L-methionine metabolism, as reported for other enzymes (16), or in the stability of these systems, as was shown for the *Pseudomonas* sp. demethiolase (21).

In the presence of L-methionine, L-ethionine had no inhibitory effect on growth, in contrast to *Saccharomyces cerevisiae* (8), whereas it increased the sMTPC. A possible explanation, considering the classical roles of L-methionine in the biological methylation processes of initiation and incorporation in protein synthesis (3) and the fact that ethionine alone is not an inducer (M. Ferchichi, D. Hemme, and M. Nardi, unpublished data), ethionine could—via competition with methionine in these processes—cause greater availability of intracellular methionine for induction. This is consistent with the possibility that the intracellular concentration of L-methionine plays a key role in the amplitude of methanethiol production.

The decreased sMTPC obtained at alkaline pH in noncontrolled cultures was not due to alkalinization, since the sMTPC was higher at a constant pH of 9 than at a constant neutral pH. This latter result may be due to the fact that intracellular pH increases with an increase of the extracellular pH, as has been reported in some other alkaline-tolerant bacteria (18). Uptake of L-methionine could not be involved, since it was lower at pH 9.0 (9 nkat g^{-1}) than at pH 7.0 (18 nkat g^{-1}).

B. linens CNRZ 918 is an alkaline-tolerant strain, as was shown by its ability to grow at constant pH 9. At this alkaline pH, cocci were present and rods subsisted and divided. This influence of constant alkaline pH on cell morphology has never been reported.

Growth rates in BMM in an agitated flask and in the fermenter at 50% O_2 saturation were similar. This demonstrated that the O_2 concentration in the agitated BMM flask culture was not limiting. The changes in specific methanethiol production, however, were not similar, being lower and decreasing more rapidly and more extensively in the case of cells in the agitated flask. No pertinent explanation for this phenomenon can be given.

The presence of L-methionine in the culture medium seemed to be a prerequisite for pigment formation. The limiting oxygen concentration which also affected orange pigment production confirmed previous results (2, 23).

These results permit industrial culture conditions to be chosen which will allow optimal methanethiol-producing capacity of the bacterial biomass, resulting in food aroma improvement.

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